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Review Article

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Spatial Omics in Decoding Oral Squamous Cell Carcinoma Heterogeneity: Microenvironment Crosstalk and Multi-Omics Integration

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Abstract
Oral squamous cell carcinoma (OSCC) is a significant concern within head and neck cancers due to its high heterogeneity, which contributes to poor treatment outcomes and low survival rates in advanced stages. This review discusses how spatial omics methods are used to explore tumor heterogeneity in OSCC. It focuses on alterations in the cellular, molecular, and immune microenvironment, which are driven by cancer stem cells, stromal interactions, genetic instability, epigenetic reorganization, and metabolic reprogramming. The focus of heterogeneity is given on the contribution of the tumor microenvironment, such as immune cells, cancer-associated fibroblasts, and extracellular matrix remodeling, to the stimulation of progression, metastasis, and therapeutic resistance. The introduction of spatial omics technologies, including spatial transcriptomics, proteomics, and metabolomics, has revolutionized the field by preserving tissue architecture, enabling high-resolution mapping of gene expression, protein distribution, and metabolite profiles. Significant developments in spatial omics technologies are discussed, along with their use in identifying ligand-receptor networks, signaling pathways, and spatial patterns of heterogeneity in OSCC. Integration of multi-omics approaches bridges gaps among transcriptomic, proteomic, and metabolic data, facilitating the discovery of biomarkers for prognosis, immune evasion mechanisms, and precision therapies targeting epithelial-to-mesenchymal transition and immunosuppressive networks. Despite challenges in data integration, cost, and clinical translation, spatial omics holds significant promise for personalized oncology. Future directions include artificial intelligence–driven modeling to enhance diagnostic accuracy and therapeutic efficacy in the management of OSCC.

Keywords:
oral squamous cell carcinoma; tumor heterogeneity; spatial omics; spatial transcriptomics; spatial proteomics; tumor microenvironment; precision oncology

1. Introduction

Oral squamous cell carcinoma (OSCC) is a malignancy of the head and neck that commonly arises in the oral mucosa [1]. It is the most common type of oral cancer, accounting for over 90% of cases [2]. The 5-year survival rate can approach ~80% when detected at an early stage(T1). If diagnosed at the later stage (T3 or T4), the chances would decrease dramatically to 20–40% [3]. OSCC treatments include surgical intervention followed by radiotherapy, chemotherapy, and immunotherapy, with chemotherapy serving as the primary adjuvant therapy for advanced OSCC [4]. However, although chemotherapy can improve survival rates, it often fails to achieve satisfactory results due to intrinsic and extrinsic drug resistance, low target specificity, and severe adverse drug reactions [5–8].

Tumor heterogeneity was recognized and proposed over 40 years ago [9,10]. Cancers may consist of multiple clonal subpopulations of cancer cells that differ in various characteristics, making all tumors inherently heterogeneous. Although heterogeneity can be inter-tumor or intra-tumor, this review focuses on intra-tumor heterogeneity unless stated otherwise, given its implications for personalized

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therapy. The pivotal role of intra-tumor heterogeneity in prognostic indicators and for methods to individualize therapy has rendered it a longstanding focal point of scholarly attention and discourse [9,11]. The existence of tumor heterogeneity necessitates a shift from our current static treatment strategies, tailoring therapies to target the specific characteristics of different cancer cell clones.

Spatial omics technologies are increasingly adopted in cancer research. While conventional bulk and single-cell sequencing have identified key mutations and cell types in tumors like OSCC, they lack the critical spatial information on where these elements exist within the tumor ecosystem, as life processes unfold in three-dimensional space, even in simple organisms or single tissues.

This review will demonstrate that spatial omics provides the missing dimension, visualizing direct interactions between cancer cells and their microenvironment to help us understand OSCC progression and therapeutic resistance that were previously less accessible. The discussion begins with foundational techniques and progresses to state-of-the-art methods. It highlights the achievements attained and explores the potential clinical applications of currently popular multi-spatial omics technologies in the study of OSCC heterogeneity. Regarding tumor heterogeneity in OSCC, the focus then shifts to how spatial omics technologies uncover tumor behavior. This includes elucidating the underlying pathways and molecular mechanisms, as well as offering potential solutions to clinical challenges. For spatial multi-omics, both Next-Generation Sequencing (NGS)-based and imaging-based methods are highlighted. The discussion concludes by addressing current challenges and providing an outlook on future directions.

2. Tumor Heterogeneity in OSCC

Tumor heterogeneity is a major factor contributing to suboptimal treatment outcomes, which is equally applicable to OSCC [12]. To minimize or eliminate the impact of this challenge on treatment strategies, a clear understanding of tumor heterogeneity is essential.

2.1. Overview of Tumor Heterogeneity

Tumor heterogeneity exists concurrently across both temporal and spatial dimensions (Figure 1A). Temporally, the distribution of cells within tumors shifts dynamically across progression stages, generating heterogeneity. Spatially, cells exhibit heterogeneity at different tumor locations, exemplified by contrasting distributions in anaerobic central regions versus well-perfused peripheral areas. Tumor heterogeneity spans epigenetic, transcriptional, phenotypic, secretory, and metabolic variation, among others [13–15]. Heterogeneity is primarily driven by two major forces: genomic instability and natural selection. It is also mediated by other factors, including epigenetic modifications and the tumor microenvironment (TME) [15,16]. However, not all clones will be able to gain genetic changes related to the phenotypes that are favorable to survival by inheritance. Under the pressure of natural selection, only a portion of these cells becomes the main constituents of the tumor (Figure 1B).

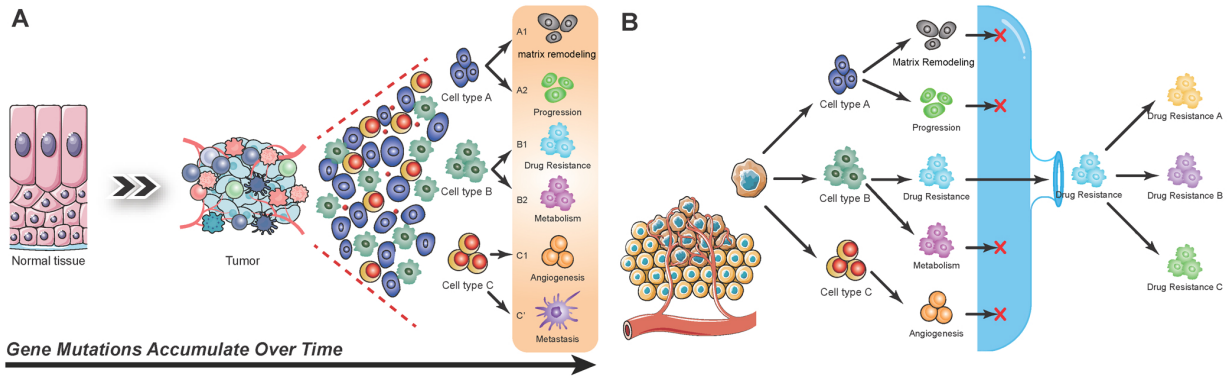

Figure 1: The formation and evolution of tumor heterogeneity. (**A**) Tumor heterogeneity in time and space. Spatial heterogeneity: diverse cell populations in the orange box vs. earlier stages. Temporal heterogeneity: positional, morphological, and functional variations among cells in the box. Horizontal axis: tumor progression over time. (**B**) Bottleneck effects in tumor progression. External selection pressures, such as radiotherapy or drug therapy, create a "bottleneck effect," allowing only specific cell subpopulations with advantageous phenotypes to survive. For example, drug-resistant cells gain a selective advantage, gradually increasing their proportion within the tumor cell population.

2025, Vol. 2, Article ID. 2025.0018
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2.2. Cellular Heterogeneity

Cellular heterogeneity is a fundamental feature of many cancers, including OSCC. Understanding it requires first examining its origins. Although no clear consensus has been reached, cancer stem cells (CSCs) have been identified as potential contributors. When gene abnormalities accumulate in somatic stem cells, due to carcinogenic exposures like tobacco, alcohol, or Human Papillomavirus (HPV), transformation into CSCs starts by undergoing procedures like epithelial-to-mesenchymal transition (EMT) [17]. They are believed to initiate the carcinogenic process, drive the progression, and promote the acquisition of heterogeneity, regardless of the manner in which CSCs proliferate [10,18–20].

However, these transformed stem cells cannot act alone. Certain seemingly "normal" cells or tissue populations present in healthy tissues are recruited and activated to become part of the tumor microenvironment (TME), thereby promoting further tumor progression [21]. The role of endothelial cells in TME in promoting tumor angiogenesis has long been acknowledged. However, the contribution of stromal cells to the hallmarks of cancer, and consequently to the fundamental nature of the disease, should not be underestimated. The stromal components of the tumor microenvironment (TME) can be broadly classified into three major categories: angiogenic vascular cells (AVCs), infiltrating immune cells (IICs), and cancer-associated fibroblasts (CAFs).

The importance of cells of these types to TME development, tumor progression, and therapeutic outcome has been recognized for their complex interactions with cancer cells and other stromal components [22]. In the case of CAFs, evidence suggests that they can interact with CSCs in OSCC, thereby promoting tumor progression [23]. Other studies suggest that CAFs' secretion also plays a crucial role in tumor drug resistance acquisition [24]. CAFs may trigger tumor recurrence by disrupting OSCC cell dormancy, and can also lead to metastasis through EMT mechanisms [25,26]. When infiltrating immune cells (IICs) differentiate into tumor-associated macrophages (TAMs), studies have demonstrated their role in interacting with tumor cells, including CSCs, and promoting tumorigenesis within the OSCC TME [27,28]. Tumorigenesis is associated with TAM polarization, and the underlying key factors and mechanisms have been identified. Gene *Thbs1* in oxidative stress mediates M1-like TAMs polarization, whereas SOAT1 promotes M2-like polarization [29,30]. These findings reveal potential therapeutic benefits and contribute to the development of new therapies for OSCC.

2.3. Molecular Heterogeneity

Tumor molecular heterogeneity refers to the variability in molecular profiles, including genetic mutations, epigenetic modifications, transcriptomic patterns, proteomic alterations, and metabolic differences, observed among cancer cells [31]. Knowledge of molecular heterogeneity helps to identify intrinsic subpopulations within OSCC that exhibit distinct functional behaviors. As a result, prognoses and responses to therapy can vary among these subpopulations. The understanding of the specialized mechanisms and processes within the various tumor subpopulations may provide hope towards personalized treatment approaches, also in the case of patients with OSCC [32,33].

Using spatial transcriptomics and spatial metabolomics, transcriptomic and metabolic landscapes can be profiled within intact tissue. The homogeneous and heterogeneous mechanisms of the origin of OSCC have been clarified by research‘ in the field of transcriptional and metabolomic changes in the progression from oral precancerous lesions to OSCC [34,35]. Specific expression patterns can be observed in key enzyme molecules related to lipid, amino acid, and glucose metabolism, indicating metabolic reprogramming in OSCC [36]. Therapies targeting metabolic pathways may also be effective against tumors [37].

Epigenetic modifications are also a key aspect of molecular heterogeneity. Modifications to histones and DNA methylation/demethylation can upregulate or suppress the expression of proto-oncogenes and tumor suppressor genes, thereby influencing perineural invasion (PNI) and contributing to adverse tumor prognoses [38]. Research using multi-omics approaches has demonstrated that the oral microbiota can contribute to OSCC development by altering promoter methylation patterns and epigenetic regulation [39]. It has also been demonstrated that CAFs can regulate DNA methylation levels and promote angiogenesis in OSCC [40]. MicroRNAs (miRNAs or miRs), a prevalent class of short non-coding RNAs, play crucial regulatory roles in epigenetics. As epigenetic modulators, miRNAs can bind to complementary target sequences in mRNA, induce mRNA degradation, and interfere with translation, thereby preventing or altering the production of the corresponding protein without modifying the underlying gene sequences [41]. A novel circRNA-miRNA-mRNA regulatory axis, which significantly contributes to oral cancer progression and malignancy, has been identified through omics profiling [42].

2.4. Immune Microenvironment Heterogeneity

The tumor immune microenvironment (TIME) of OSCC is characterized by changes in immune factors (tumor and matrix), checkpoints, and cell populations that promote immunosuppression, thereby facilitating tumor immune evasion [43,44]. The principal regulatory molecules within the immune microenvironment are secreted by both tumor cells and heterogeneous immune cells, modulating tumor behavior through their interactions while concurrently remodeling the extracellular matrix (ECM) and influencing the morphology and function of the immune microenvironment. These critical immune cells, along with their associated receptors and signaling pathways, exhibit heterogeneous expression patterns within tumors. This heterogeneity presents both challenges and opportunities for cancer therapeutics.

Among these signaling pathways, certain key molecules have garnered particular attention, such as Interleukin-6 (IL-6) and IL-10, Programmed Death-Ligand 1 (PD-L1), and Transforming Growth Factor-β (TGF-β) [45]. The impact of TGF-β on TIME in OSCC has been extensively studied. It is a multifunctional cytokine secreted by CAFs, TAMs, and regulatory T cells (Tregs) within the OSCC TME [46]. By regulating the behavior of downstream cells within the tumor microenvironment (TME), such as the M2 polarization of macrophages, it has been reported to exert immunosuppressive effects and promote tumor immune evasion and progression in OSCC at different stages [47–49]. Meanwhile, it is demonstrated that the formation and remodeling of the ECM are also modulated by TGF-β [50]. This process, which is normally intended for tissue repair, can become dysregulated during chronic and persistent inflammation. Excessive accumulation of collagen, fibronectin, hyaluronic acid, and proteoglycans may occur, thereby creating conditions conducive to tumorigenesis [50,51]. Concurrently, TGF-β exhibits heterogeneous secretion across different tumor subclones and stromal compartments, thereby mediating pathways such as EMT and tumor-ECM communication. These pathways are widely recognized as key potential therapeutic targets for tumor growth, therapeutic resistance, and invasive capacity [52–55]. Targeted inhibition of TGF-β signaling shows promise in restoring anti-tumor immunity [56].

3. Spatial Omics and Application in OSCC

OSCC is a heterogeneous malignant neoplasm characterized by complex molecular pathways and diverse genetic features, and this heterogeneity presents significant challenges for research and clinical management [57]. Traditional omics approaches suffer from the loss of spatial information [58]. Therefore, spatial omics has emerged, enabling the in situ visualization of cellular interactions and heterogeneity while preserving the spatial context of tissues, thereby elucidating how tissues function or malfunction within tumors [59].

From spatially resolved transcriptomics (Method of the Year 2020 by Nature Methods [60]) to spatial proteomics (Method of the Year 2024 by Nature Methods [61]), spatial omics has emerged as a pivotal driving force in life sciences research. Technological revolutions in Immunohistochemistry (IHC), In Situ Hybridization (ISH), and NGS-based methods are advancing spatial omics to the subcellular level, offering greater flexibility in detecting spatial patterns of gene and protein expression, genetic mutations, epigenetic marks, chromatin structure, and genome organization [59,62–65].

The general workflow involves applying specialized probes to a prepared tissue section to bind target molecules. These molecules are identified using methods such as next-generation sequencing (NGS) or mass spectrometry, and their original coordinates are used to reconstruct a detailed spatial map (Figure 2).

This spatially resolved data allows for comprehensive analysis of biomolecular abundance, regulatory pathways, and interaction networks, thereby elucidating how tissues function or malfunction within tumors. Thus, spatial omics has become one of the most powerful tools for scientific research [60,61].

From bulk omics to high-resolution spatial mapping, driven by advances in imaging and sequencing, spatial omics has branched into transcriptomics, proteomics, and metabolomics.

3.1. Spatial Transcriptomics

Spatial transcriptomics (ST) was born with the goal of mapping every gene and gene isoform, at subcellular resolution, in a whole tissue sample with spatial dimensions. Although no single technique achieves optimal performance across all metrics—sensitivity, resolution, throughput, and ease of use—desired data can still be obtained by carefully balancing the choice of methods [67].

3.1.1. Spatial Transcriptomics Strategies

(1) Next-generation sequencing (NGS)-based strategies are designed to capture transcriptomic information within tissue by tagging reads originating from different locations with short sequences carrying spatial information (spatial barcodes/IDs, similar to

2025, Vol. 2, Article ID. 2025.0018
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the labels on goods in shops). Transcriptomic information is captured within the tissue through complementary base pairing of barcodes and RNAs in tissue, and transcriptomic information is stored as the base sequence information reverse-transcribed from barcodes into cDNA. Following cDNA sequencing, the information is computationally reattributed to its original spatial location using these barcodes.

Technologies including Visium [58], Visium HD [68], Slide-seqV2 [69], and Stereo-seq (Table 1) are currently the most widely applied NGS-based strategies. These techniques each offer advantages in areas such as user-friendliness and formalin-fixed paraffin-embedded (FFPE) compatibility, or sensitivity and ultra-high resolution. This diversity requires choice for research needs, whether in clinical applications or precision studies.

These four technologies primarily rely on NGS for whole-transcriptome profiling while capturing spatial information through array or bead-based barcoding. They differ in resolution, throughput, sample compatibility, and sensitivity, with advancements focusing on higher spatial fidelity and RNA capture efficiency.

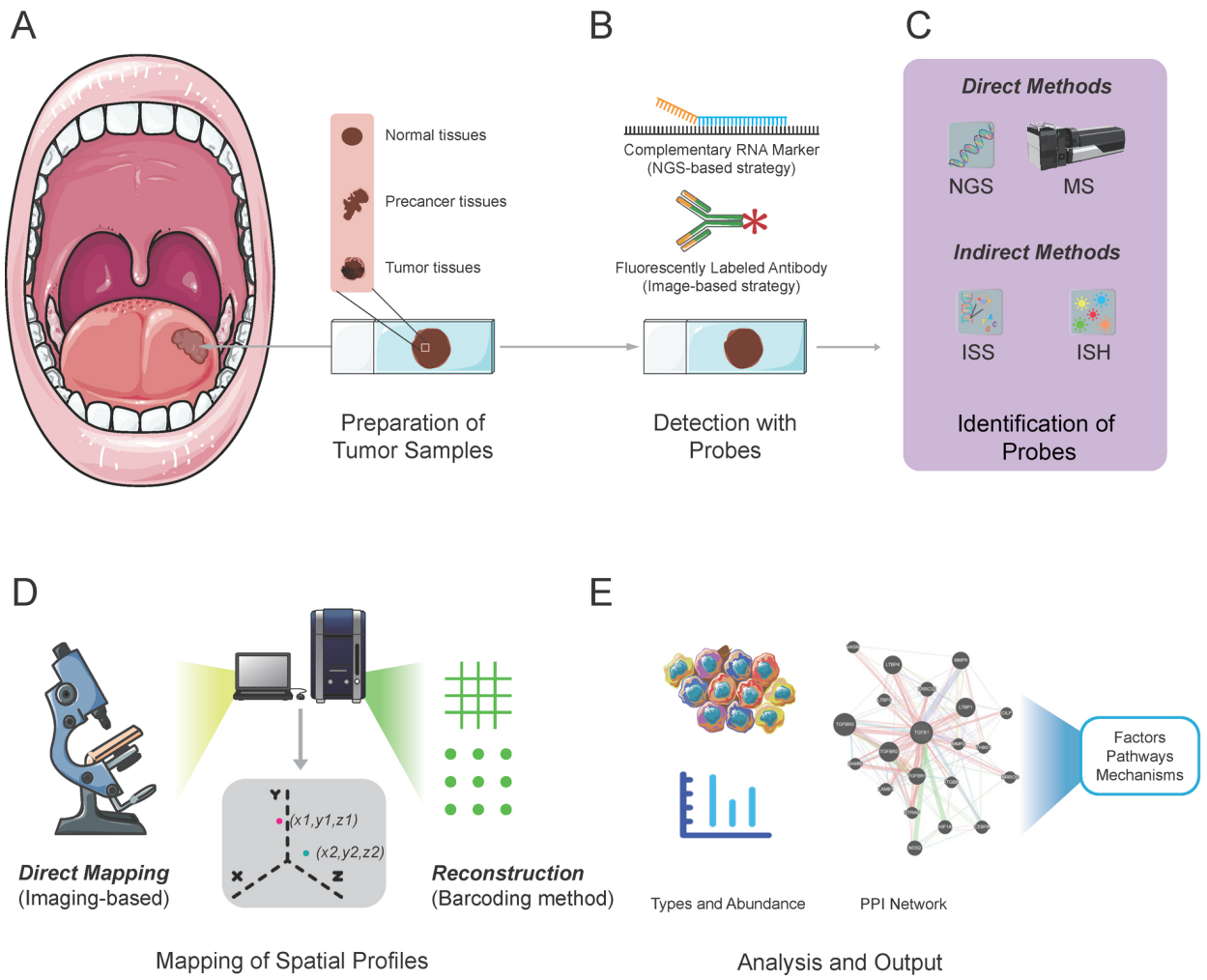

Figure 2: Workflow for Studying Tumors by Spatial Omics. (**A**) Tumor sample preparation. For studying heterogeneity, including normal/precancerous and tumor tissues is needed. (**B**) Probe-based detection of expression profiles. Oligonucleotide probes (barcode-encoded at one end) or fluorescently labeled antibodies bind specific nucleic acids, proteins, or small molecules. (**C**) Identification of bound molecules. Use next-generation sequencing (NGS) or mass spectrometry (MS) for direct detection, or indirectly via individual probe markers. (**D**) Spatial mapping. Imaging methods yield direct images; sequencing methods reconstruct patterns from pre-acquired coordinates. (**E**) Analysis and output. Quantify/validate metrics such as biomolecular abundance, regulatory functions, and protein-protein interaction (PPI) networks to uncover key factors, pathways, and mechanisms underlying tumor behavior. Note. PPI network generated using the GeneMANIA platform [66]. No additional permission required for academic use, as per GeneMANIA website.

Table 1: Comparison of sequencing-based spatial transcriptomics methods.

Platform	Visium	Visium HD	Slide-seqV2	Stereo-seq
Developer/ Company	10x Genomics	10x Genomics	Broad Institute	BGI
Principle/ Mechanism	Array-based spatial barcoding with poly-T capture for mRNA; NGS readout.	Enhanced array-based barcoding; probe-based chemistry with CytAssist for probe transfer; NGS readout.	Bead-based puck array; high-density beads with barcodes; optimized library prep for sensitivity; NGS readout.	Chip-based nanometer-scale barcoding; high-density array; NGS readout.
Spatial Resolution	55 μm feature size (spots).	2 μm square pixels (higher fidelity).	~10 μm feature size (near cellular).	0.22–1 μm feature size (nano-scale).
Throughput/ Gene Detection	Whole transcriptome (>20,000 genes); medium throughput (millions of reads per slide).	Whole transcriptome (>20,000 genes); high throughput (millions of reads improved capture).	Whole transcriptome; high sensitivity (~50% RNA capture efficiency ~45,000 UMIs per array).	Whole transcriptome (>20,000 genes); ultra-high throughput (billions of reads per cm^2).
Sample Compatibility	Fresh frozen and FFPE (with CytAssist).	FFPE; adaptations for fresh.	Fresh frozen	Fresh frozen; adaptations for FFPE.
Key Advantages	Versatile for fresh/FFPE; simple workflow; integrates with histology; no special instruments beyond NGS.	Superior spatial fidelity vs. standard Visium; minimizes diffusion; complements imaging tools; unbiased discovery.	High detection efficiency (10x over original Slide-seq); excels in rare transcript detection and trajectory analysis.	Highest resolution among sequencing-based, large-area coverage, cost-effective for scale, ideal for heterogeneous tissues.
Key Limitations	Lower resolution (multi-cellular per spot); diffusion artifacts; requires downstream deconvolution for single-cell insights.	Higher cost; requires CytAssist instrument; data processing is intensive for large datasets.	Limited to fresh tissues; bead synthesis complex; lower scalability for very large areas; computational demands for indexing.	Massive data volumes require advanced computing, sample prep complexity, and potential overkill for low-res needs.

(2) Image-based strategies rely on the combination of transcriptomic data with fluorescent labelling signals in situ without reconstruction. The primary approach involves acquiring spatial information using fluorescent labels paired with transcripts. These methods are chiefly categorized as in situ sequencing (ISS) and in situ hybridization (ISH). ISS employs in situ amplification to enhance signals, though it suffers from low sensitivity and difficulties in reading long-chain signals. ISH relies on pre-set complementary sequences to capture specific transcriptomic content. While its analyzed items are clearly defined, it is typically restricted to predefined gene panels.

The imaging-based technologies Xenium, CosMx, and STARmap PLUS [70], and MERFISH [71] (Table 2) provide true single-cell/subcellular resolution, but their respective pros and cons also require researchers to make their own choices.

These technologies focus on in situ hybridization (ISH) or sequencing (ISS) for subcellular resolution, but with targeted gene panels (hundreds to thousands). They emphasize direct visualization of transcripts in tissue context, differing in plexity, runtime, and customization. Below is a structured comparison.

Due to the significant differences between NGS-based and imaging-based strategies, researchers must make intentional choices for different study subjects. Generally, NGS-based methods provide unbiased genome-wide coverage; however, their relatively low-resolution limits them, making these methods most suitable for exploring gene expression profiles at scale and capturing the overall level of cellular expression within a given region. In contrast, imaging-based techniques such as MERFISH, which provide subcellular resolution, are better suited for hypothesis validation, for example, to precisely locate a specific subpopulation of immune cells relative to cancer-

2025, Vol. 2, Article ID. 2025.0018
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ous stem cells. However, reliance on a predefined gene panel restricts the region of interest (ROI). This situation is evolving with advances in technology. For example, the resolution of Visium HD now reaches the subcellular level, offering greater analytical capabilities, albeit at the expense of higher costs. An approach that combines the strengths of the diverse approaches might be to apply them at different stages of the research.

Table 2: Comparison of imaging-based spatial transcriptomics technologies.

Platform	Xenium	CosMx	STARmap PLUS	MERFISH
Developer/ Company	10x Genomics	NanoString	Broad Institute (Wang et al.)	Harvard/Vizgen (MERSCOPE)
Principle/ Mechanism	ISS-based with padlock probes, rolling circle amplification (RCA), and cyclic fluorescent imaging.	ISH-based with gene-specific probes and optical signatures; cyclic hybridization.	ISS-based with in situ sequencing and amplification; enhanced for larger panels.	ISH-based with multiplexed error-robust FISH; binary encoding for probes.
Spatial Resolution	Subcellular (<1 μm; transcript-level).	Subcellular (<1 μm; xyz coordinates).	Subcellular (~1 μm; 3D capable).	Subcellular (<1 μm; high-plex imaging).
Throughput/Gene Detection	Targeted (up to 5K genes); high cell throughput (~100,000–millions per run).	Targeted (up to 6K genes, including non-coding); medium-high throughput (~100,000 cells)	Targeted (~1K genes); very high cell throughput (up to millions).	Targeted (hundreds to 1K genes; customizable up to 1K).
Sample Compatibility	Fresh frozen and FFPE.	Fresh frozen and FFPE.	Fresh frozen tissue sections.	Fresh frozen and FFPE.
Key Advantages	Large sample area (full slide); shorter runtimes (~half of CosMx); reliable, high-quality data; integrates with Visium.	Larger panels; good for multi-tissue; automatic data to cloud (AtoMx); identifies major cell types well.	High plex for ISS; excellent for 3D brain mapping; integrates with spatial imaging; cost-effective for large tissues.	Error-robust (binary coding); fully customizable panels (no design fees); open platform for any species; good for subtypes despite lower counts.
Key Limitations	Limited to pre-designed panels (human/mouse focus); longer prep; data access via instrument.	Longer runtimes (2x Xenium); preselect ROIs (limited area); higher noise potential; custom design fees; data export required via subscription.	Limited public data/customization; lower plex vs. ISH peers; requires specialized analysis for massive datasets.	Lower sensitivity/specificity in some cases; limited to predefined panels; longer imaging times; challenging annotation without markers.

3.1.2. Application of Spatial Transcriptomics in OSCC

Spatial transcriptomics technology captures cellular transcriptomic profiles while preserving tissue architecture, thereby revealing cellular behaviors and heterogeneity in OSCC. These insights can subsequently be translated into guidance for clinical applications.

The spatial distribution of gene expression patterns, such as differences between upregulated genes in the tumor core and leading-edge architectures, reveals the spatial underpinnings of heterogeneity in the tumor and TME, allowing prediction of drug targets, evaluation of therapeutic responses, and patient survival rates [72].

Identification of cellular subpopulations and their composition, including cancer cells, immune infiltrating cells, and stromal components such as tumor-associated fibroblasts (CAFs) [35]. CAF subsets contribute to tumorigenesis, metastasis, and therapy resistance by remodeling the ECM, promoting angiogenesis, secreting growth factors and cytokines, and suppressing anti-tumor immunity [73]. Researches have also demonstrated the tumor-suppressing role of other CAF subsets, suggesting that cellular heterogeneity provides diverse and complex patterns for tumor

function [74]. In OSCC, spatial transcriptomics has found that CAFs can interact with epithelial cells which located at the tumor's stromal front, causing oxidative stress overload [75]. It is also reported that CAFs promote cancer cell metastasis through EMT [25]. The in-depth exploration of CAFs is not just a glimpse of the tumor's biological basis. Furthermore, by uncovering and comparing various signaling pathways, promising targets for improving patient prognosis can be identified. By understanding the mechanisms of CAF action, characteristic diagnostic clues will be recognized, thereby contributing to the development of personalized treatments in precision medicine.

Ligand–receptor interaction networks, which highlight crosstalk between tumor cells and microenvironmental cells, form the basis for tumor communication with the surrounding environment [76]. A protein kinase RNA-like ER kinase (PERK)-signaling cascade is highlighted, as a key sensor in the unfolded protein response, in driving metabolic reprogramming and epigenetic changes in TAMs via the PERK-ATF4-PSAT1 axis [77]. By targeting the unfolded protein response relevant PERK/activating transcription factor 4 (ATF4) signaling pathway, tumor control in both primary tumors and lymph node metastases may be achieved, and the direct identification of these potential targets provides guidance for the development of targeted therapeutics [78].

3.2. Spatial Proteomics

Spatial proteomics enables the measurement of protein abundance, distribution, and interactions within tissue sections, with characteristics similar to those of spatial transcriptomics. In OSCC, it revealed tumor heterogeneity, tumor-stroma interactions, and signaling pathway activations, offering insights into mechanisms of progression, metastasis, and therapeutic resistance [79].

3.2.1. Spatial Proteomics Strategies

The concept of imaging-based strategies, previously introduced in transcriptomics, is similarly applied to proteomics. Compared to the short RNA sequences used in transcriptomics, referred to as Barcodes, the approach here relies more on the antibody (as the binding component) and fluorescent protein molecules (as the indicator). This involves using ISS/ISH techniques with antibody- or aptamer-based probes conjugated to fluorescent, oligonucleotide, or metal tags to detect proteins.

Iterative fluorescence methods enable multiplexed protein imaging through cyclic antibody staining, imaging, and signal quenching/bleaching to reuse channels. The binding parts (antibodies) are repeatedly replaced, but the biological information is retained for measurement. Owing to distinct marker characteristics (such as fluorescent color) and powerful computational processing capabilities, a single experimental round can detect a substantial number of antibodies. IBEX employs bleaching across>65 parameters with quick 2–5-days runs and open-source flexibility, though it may compromise tissue integrity. CycIF combines bleaching with chemical quenching for cost-effective, non-destructive workflows on FFPE tissues, but risks cumulative signal loss.

Oligonucleotide-tagged methods, such as the upgraded CODEX platform PhenoCycler-Fusion (PCF), barcode antibodies for hybridization and detection, supporting >100 biomarkers while preserving samples for multi-omics integration. PCF is an automated, high-plex phenotyping technology that requires lengthy, multi-day protocols and higher costs. Mass spectrometry (MS)-based spatial proteomics maps protein distributions by labeling antibodies with stable isotopes or metals, ablating tissue regions, and analyzing ionized peptides/proteins using MS for quantitative, interference-free detection at single-cell resolution (~1 μm).

Imaging Mass Cytometry (IMC) functions as a scanner (such as the one in the supermarket) that uses a UV laser to carefully burn off tiny spots on tissue samples labeled with special metal-tagged antibodies. These measurements are then obtained using a process known as time-of-flight mass spectrometry, which can analyze up to ~40 markers. Its benefits include strong capability to analyze TME, high reproducibility, and easy data interpretation. Disadvantages include a fixed resolution of ~1 μm, inability to rescan samples, and the high cost of equipment.

In Multiplexed Ion Beam Imaging (MIBI), particles on metal-tagged tissue samples are gently knocked off by a focused beam of ions, and the resulting secondary ions can be analyzed, enabling tunable resolution (30–50 nm in HD mode) and multiple scans (~100 targets). The main advantages include high subcellular resolution, the ability to see whole-tissue images, and reduced artifacts in fluorescent images. Disadvantages include long acquisition times, the high cost of tags and instruments, and increased data complexity.

3.2.2. Application of Spatial Proteomics in OSCC

Spatial proteomics has evolved into one of the most powerful tools in precision oncology for OSCC. Preserving spatial context at near-single-cell resolution allows clinicians and researchers to answer critical real-world questions that traditional bulk proteomics or IHC cannot address: Which proteins are driving lymph node metastasis in this patient's tumor? Why does one region of the tumor respond to anti-PD-1 while the adjacent region is completely

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resistant? How is the tumor stroma (especially CAFs) being reprogrammed to promote invasion and treatment resistance? Can we identify patients who will truly benefit from immune checkpoint inhibitors or EGFR-targeted therapies based on spatially resolved biomarker patterns?

In the field of spatial mapping of protein markers and signaling pathway activation profiles, new spatial proteomics technologies are continually being developed and applied. Studies on OSCC and head and neck squamous cell carcinoma (HNSCC) have provided direct clinical insights.

For instance, one study applied digital spatial profiling with an immune pathway panel to mucosal HNSCC samples. It demonstrated regional differences in immune checkpoint molecules such as PD-1, PD-L1, LAG-3, and TIM-3, activation markers including Granzyme B and Ki-67, and immunosuppressive cytokines across the tumor core, invasive margin, and stroma [80]. This helped explain varying responses to PD-1 blockade by revealing hot, cold, and excluded immune phenotypes within the same tumor.

Another investigation conducted proteogenomic and spatial analysis on primary OSCC and matched metastatic lymph nodes. It found that extracellular matrix remodeling proteins, including COL11A1, THBS2, and Tenascin-C, secreted by activated CAFs create premetastatic niches, particularly in cases with lymph node involvement. These matrix signatures were linked to cetuximab resistance and poor prognosis [25].

Furthermore, a study integrated mass spectrometry-based spatial proteomics with single-cell transcriptomics in a large cohort of OSCC patients. It successfully divided patients into three practical subgroups: an immunogenic type with high PD-L1, CD8, and IFN-γ signaling that responds well to anti-PD-1; a stromal-rich or desmoplastic type with elevated TGF-β and CAF markers that resists immunotherapy but may benefit from TGF-β inhibitors; and a metabolic or Epidermal Growth Factor Receptor (EGFR)-driven type suitable for EGFR monoclonal antibodies or combined approaches [81].

In addition, emerging panoramic ultra-high-resolution techniques have been employed in glioma samples and are now being adapted to OSCC. These techniques map gradients of EGFR, phospho-EGFR, and related signaling from the tumor center to the invasive front, potentially aiding in predicting responses to anti-EGFR therapy prior to treatment [82].

Moreover, spatial proteomics reveals that TP53 gain-of-function mutations in non-HPV OSCC actively shape an immunosuppressive TIME by downregulating interferon-stimulated genes and T-cell chemokines. This explains the disappointing response of TP53-mutated OSCC to checkpoint inhibitors and supports ongoing trials combining PD-1 blockade with STING or type I IFN pathway agonists [83].

3.3. Spatial Metabolomics

Spatial metabolomics is a research method designed to meet the need for assessing metabolites and metabolic conversion rates, given the fact that metabolic rewiring is a critical component of tumor progression [36].

3.3.1. Spatial Metabolomics Strategy

Mass spectrometry imaging (MSI) is a label-free technique that enables the spatial mapping of hundreds of metabolites and drugs directly from a tissue section [84]. Nowadays, MS techniques are commonplace in cancer research. Matrix-assisted laser desorption ionization (MALDI) has been applied for imaging peptides and proteins in biological samples for many years [85,86]. Subsequently, the development of desorption electrospray ionization (DESI) technology has established MSI as a powerful technique, capable of spatial visualization of molecules like amino acids, proteins, lipids, polysaccharides, and oligonucleotides [87,88]. Beyond the fundamental identification and quantification of metabolites, MS can also use stable non-radioactive isotope labeling (e.g., ^{13}C, ^{2}H, or ^{15}N) to assess the impact of nutrients and metabolic pathways on specific observed metabolic changes [86,89]. Moreover, ideas for improving MS continue to be proposed and applied. Previously, the integration of 3D MALDI MSI data with H&E and IHC images was reconstructed from 162 consecutive human OSCC tissues, providing better insight into tumor functional heterogeneity [90].

3.3.2. Application of Spatial Metabolomics in OSCC

For OSCC, metabolomics-based assessment of tumor heterogeneity can assist clinical treatment across multiple critical stages, including prevention, diagnosis, therapy, and prognosis. The spatial metabolomics characteristics of oral submucous fibrosis (OSF)-derived OSCC reveal metabolic reprogramming procedure, confirming remarkable metabolic differences between amino acid metabolism (in malignant epithelial region, associated with poorer prognosis in patients), galactose metabolism, lipid metabolism, and ABC transporters (in OSF and stroma regions) [35]. During diagnosis, spatial metabolomics can also distinguish tumor margins from normal mucosa by detecting variations in characteristic amino acids, fatty acids, and fatty acid esters of hydroxy fatty acids, thereby aiding pathological diagnosis and surgical planning [91,92]. Meanwhile, procollagen-lysine, 2-

oxoglutarate 5-dioxygenase 2 (PLOD2), a key enzyme in the generation of collagen cross-links, has been demonstrated as a poor prognostic biomarker for OSCC and may affect metastasis through the EMT pathway, as examined by MS and IHC [93]. Additionally, distinct metabolic profiles were observed across different regions of OSCC tumors, revealing elevated metabolic rates between the tumor center and the invasive margin. This suggests that activated purinergic signaling may be a potential target for therapeutic strategies targeting the TME [94].

3.4. More Spatial Omics Technologies and Applications

The three spatial omics technologies discussed in this review are not all-inclusive; other valuable spatial omics approaches exist for uncovering additional insights into OSCC. Spatial epigenomics and microbiomics offer profound value in OSCC research by unveiling spatially resolved molecular and microbial landscapes that drive tumor heterogeneity, progression, and therapeutic resistance.

Spatial epigenomics, leveraging technologies such as spatial Assay for Transposase-Accessible Chromatin with high-throughput sequencing (ATAC-seq) and Cleavage Under Targets and Tagmentation (CUT&Tag), brings chromatin accessibility to the field and spatially maps epigenetic modifications like DNA methylation across tumor genes, offering new opportunities to investigate epigenetic regulation, cell function, and fate decision in normal physiological processes and pathological mechanisms [95,96].

Spatial microbiomics has evolved from traditional methods and 16S rRNA sequencing to metagenomics and spatial genomics. Spatial transcriptomics techniques, such as 10x Visium and GeoMx digital spatial profiling, can be combined to reveal spatial, cellular, and molecular host–microbe interactions [97]. It described the patterns of microbial community distribution in the OSCC TME, highlighting dysbiotic interactions that promote inflammation, immune evasion, and metastasis. These interactions can inform microbiome-modulating interventions to enhance immunotherapy efficacy and prognosis.

A combination of these technologies enables precise investigation of tumor ecosystems, particularly in heterogeneous malignancies like OSCC.

4. Multi-Omics and Application

4.1. Integration of Multiple Tumor Analysis Methods

The technologies we employ to detect tumor heterogeneity are continually evolving. On a cellular population basis, we can examine the mean gene expression phenotype of whole tissues using bulk analysis [98]. On the other hand, analysis of single cells enables us to distinguish between cell subtypes [99]. Today, spatial omics enables us to understand the spatial patterns of gene expression within cells (or of cell-ECM interactions) at the subcellular level. Although these levels of omics exhibit a progression in terms of resolution, each of them has its own benefits and complements functionally. The integration of these multi-level approaches often yields synergistic effects where the whole is greater than the sum of its parts.

This principle also applies to spatial omics technologies. Spatial transcriptomics reveals potential functional instructions, which could be seen as the cell's "action plan"; Spatial proteomics maps cellular effectors, analyzing the "actual execution" of cellular functions. What sparks might their collision create?

As discussed above, the technical methods used in spatial proteomics and transcriptomics are similar, making parallel analysis possible. In practice, platforms like Nanostring GeoMx DSP can already profile expression of RNA and protein from distinct tissue compartments and cell populations, creating more convenience for the application of multi-omics methods [100].

Moreover, integration with epigenomics helps elucidate principles of gene regulation; combining spatial metabolomics provides a more detailed view of biological transformations; and incorporating microbiomics enables understanding of how external factors influence the TME. Spatial heterogeneity in those fields illustrates bottlenecks and regulatory mismatches in the process of translating genetic instructions into functional protein expression and onward to metabolism, a process of genotype-to-phenotype.

To summarize, single-cell level methods are limited to provide information of just one element of the biological processes, compared to multi-omics that can provide a multidimensional view of different facets of the biological processes. Multi-omics help us gain a systems-level perspective of biological phenomena and processes

4.2. Application of Multi-Omics in OSCC

Integration of multi-omics not only bridges the information gap between transcription, translation, and effector functions but also plays a crucial role in analyzing TME, discovering biomarkers, and designing precision treatment strategies.

4.2.1. Exploration of TME

Today, innovative multi-omics technologies can provide deep insights into the TME, focusing on intercellu-

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lar and cell-matrix interactions, matrix remodeling, and metabolic status. By bulk and single-cell omics screening the region of interest and uncovering tumor behaviors, we can identify distinct cellular subtypes in OSCC, which may exhibit distinct levels of genetic variation, malignancy, and poor prognosis.

Subsequently, the Caveolin-1 (CAV-1+) epithelial subtype can be shown by spatial omics to interact with T cells through the Nectin Cell Adhesion Molecule 1-Cluster of Differentiation 96 (NECTIN1-CD96) signaling network, which could cause immune evasion [101]. Furthermore, spatial omics methods have been employed to characterize the EMT process, a key mechanism in oral cancer progression, invasion, and metastasis [25]. Key cells, pathways, and molecules that influence EMT activation through oxidative stress signaling were revealed [75].

4.2.2. Identification of Biomarkers and Signaling Pathways

Overall, the focus of tumor research remains on developing, evaluating, and refining treatment strategies, which inherently rely on the identification of specific signaling molecules or pathways.

Clinicians can tackle real-world challenges in precision therapy, such as how to overcome resistance to ferroptosis inducers or metal-dependent cell death pathways that drive cancer stem cell survival and metastasis. One validated CD44-targeted therapy using mP6/Rg3 micelles promotes ferroptosis in cancer stem cells by inhibiting ATP Binding Cassette Subfamily B Member 1 (ABCB1), improving OSCC pathology [102]. The other confirmed that this mechanism correlates with lymph node metastasis and resistance to both immunotherapy and anti-*EGFR* targeted therapies. This research further identified Doramapimod as a potential therapeutic candidate [103].

Other questions arise regarding why some OSCC patients develop rapid resistance to immunotherapy, anti-EGFR agents, or cisplatin, and how this resistance can be predicted before treatment. Research on cisplatin-resistant cells has revealed associations with EMT, inflammatory signaling, and metabolic adaptation, suggesting adjunctive strategies such as allyl isothiocyanate to inhibit proliferation and induce apoptosis, while also linking metal-dependent resistance to these therapeutic failures [104].

Which immune or metabolic regulators in TME could serve as actionable targets to enhance invasion control and overall survival, as demonstrated in a study on the C-C Motif Chemokine Ligand 26 (CCL26) protein within TME, indicating its role in tumor cell invasion, while also investigating its interactions with other immune cells [105]. Additionally, the epigenetic factor miR-181a-5p and its regulatory pathways in lipid metabolism were also identified through multi-omics analysis, suggesting its role in maintaining oral mucosal homeostasis [106].

To enhance the efficacy of combination therapy, spatially based prognostic models could be developed and individually refined to achieve optimal outcomes. A recent systematic analysis examined six major mechanisms in OSCC: inflammation, proliferative signaling, immune regulation, oxidative stress, angiogenesis, and epigenetic regulation. The study examined relevant signaling pathways and therapeutic targets and evaluated the efficacy of various treatment approaches [107]. It also found that Transcription Factor 7–positive (TCF7+) T cells were significantly associated with increased survival rates in OSCC, contributing to the assessment and optimization of tumor therapy efficacy [108].

In general, multi-omics is transforming the field from general-level research to personalized care, decoding TME dynamics, speeding up the development of biomarker-based interventions, overcoming resistance, and improving patient prognosis.

5. Conclusions and Perspectives

In conclusion, spatial omics is an important tool for analyzing OSCC and other cancers, providing a clear picture of tumor heterogeneity. Regarding TME-tumor cell interactions, OSCC research has developed a mature workflow model for research on similar diseases such as head and neck cancer, lung cancer, and breast cancer. In the protein/gene expression field, it identifies diverse expression patterns and helps identify biomarkers for prognosis and immunotherapy in OSCC. Regarding pathways, it reveals key processes such as EMT-oxidative stress and PERK-ATF4 in OSCC growth, suggesting therapies that could target metabolic alterations in other solid cancer types. These approaches have been widely used in oncology and other fields and will certainly be applied to areas of research that have not yet been examined. In the oral cavity, metabolomics and spatial proteomics can be used to study salivary gland secretion patterns, which can guide the treatment of diseases such as Sjögren's syndrome. The complexity of the oral microbiome environment can be compared to that of the TME, suggesting that spatial multi-omics can be useful for studying its heterogeneity.

Despite ongoing progress, spatial omics faces significant challenges in clinical translation. Spatial omics is characterized by flaws, such as a lack of standardization, which make results difficult to compare across studies or to implement in clinical settings. In oncology, validation of spatial biomarkers for OSCC immunotherapy or prog-

nosis requires further trials, yet due to the high cost and complexity of the data, this cannot be achieved.

For standardization, the challenge is to integrate data across scales and modalities. Vertically, combining bulk omics (which averages signals across tissues), single-cell omics (which captures individual-cell details but loses location), and spatial omics (which adds positional context) is challenging. The disparities between resolutions and data types make it difficult to match multi-omics approaches, leading to the inability to align them without losing accuracy or introducing bias. Horizontally, the integration of transcriptomics (gene expression), proteomics (protein levels), metabolomics (metabolite profiles), and epigenomics (DNA modifications) is complicated by the use of different data types and their distinct dimensions and biological layers. This scenario requires complex computational models to provide a mutually consistent perspective on OSCC heterogeneity.

In particular, spatial transcriptomics faces low throughput and high costs as major problems. Experiments such as 10x Visium or MERFISH can cost thousands of dollars per sample, which prevents routine experiments or large cohorts. Also, throughput is limited, and many techniques process hundreds to thousands of genes or cells at once and, therefore, are slower than bulk sequencing for performing extensive studies. These issues complicate scaling up OSCC research because labs cannot afford the costs and time required.

Spatial omics needs to bridge the gap between innovative science and everyday clinical use by overcoming significant challenges of standardization and cost-effectiveness. Implementing robust standardization is paramount. This includes implementing comprehensive Standard Operating Procedures (SOPs) for sample processing, particularly for clinically relevant FFPE tissues, to ensure data consistency. In addition, cross-study comparisons and meta-analyses require adopting a common data format that incorporates expression matrices, spatial coordinates, and histology images.

At the same time, it is critical to make it more affordable and scalable for more extensive use. Feasible measures include developing lower-cost, non-proprietary technologies for probe synthesis to reduce reagent costs. By leveraging sample multiplexing methods, such as Tissue Microarrays (TMAs), one can analyze a large number of samples in a single run, thereby reducing costs per sample by a significant margin. Moreover, automating laborious processes through robotics can reduce reagent waste and maximize throughput, making large-scale clinical studies more feasible. This approach also brings the transition of the technology toward diagnostic applications closer, as demonstrated by refinements in the Assist version of the Visium platform.

Moving forward, spatial omics, being an exciting new technology, will allow us to venture into more frontiers that have not been well charted. Combining multi-omics and AI analysis will enable real-time spatial mapping and dynamic monitoring of individual cells, revealing the heterogeneity of OSCC tumors and mechanisms of TME evolution.

List of Abbreviations

ABCB1	ATP Binding Cassette Subfamily B Member 1
ABC transporters	ATP-Binding Cassette Transporters
ATAC-seq	Assay for Transposase-Accessible Chromatin with Sequencing
ATF4	Activating Transcription Factor 4
AVCs	Angiogenic Vascular Cells
CAFs	Cancer-Associated Fibroblasts
CAV1+	Caveolin-1 Positive
CCL26	C-C Motif Chemokine Ligand 26
CD44/96	Cluster of Differentiation 44/96
CODEX	Co-Detection by Indexing
CRISPR/Cas9	Clustered Regularly Interspaced Short Palindromic Repeats / CRISPR-Associated Protein 9
CSCs	Cancer Stem Cells
CUT&Tag	Cleavage Under Targets and Tagmentation
DESI	Desorption Electrospray Ionization
ECM	Extracellular Matrix
EGFR	Epidermal Growth Factor Receptor
EMT	Epithelial-to-Mesenchymal Transition
ER	Endoplasmic Reticulum
FFPE	Formalin-Fixed Paraffin-Embedded
FISH	Fluorescence In Situ Hybridization
GLUT-1	Glucose Transporter 1
H&E	Hematoxylin and Eosin
HIF-1α	Hypoxia-Inducible Factor 1-Alpha
HNSCC	Head and Neck Squamous Cell Carcinoma
HPV	Human Papillomavirus
IHC	Immunohistochemistry
IICs	Infiltrating Immune Cells
IL-6/10	Interleukin-6/10
IMC	Imaging Mass Cytometry
IPA	Immuno-Oncology Proteome Assay
ISH	In Situ Hybridization
ISS	In Situ Sequencing
MALDI	Matrix-Assisted Laser Desorption Ionization
MERFISH	Multiplexed Error-Robust Fluorescence In Situ Hybridization

MIBI	Multiplexed Ion Beam Imaging
miRNAs/miRs	MicroRNAs
MS	Mass Spectrometry
MSI	Mass Spectrometry Imaging
NECTIN1	Nectin Cell Adhesion Molecule 1
NGS	Next-Generation Sequencing
OSCC	Oral Squamous Cell Carcinoma
OSF	Oral Submucous Fibrosis
PCF	PhenoCycler-Fusion
PD-1	Programmed Cell Death Protein 1
PD-L1	Programmed Death-Ligand 1
PERK	Protein Kinase RNA-like Endoplasmic Reticulum Kinase
PLOD2	Procollagen-Lysine, 2-Oxoglutarate 5-Dioxygenase 2
PNI	Perineural Invasion
PPAR	Peroxisome Proliferator-Activated Receptor
PSAT1	Phosphoserine Aminotransferase 1
PSERP	Panoramic Spatial Enhanced Resolution Proteomics
scATAC-seq	Single-Cell Assay for Transposase-Accessible Chromatin with Sequencing
scRNA-seq	Single-Cell RNA Sequencing
SM	Spatial Metabolomics
ST	Spatial Transcriptomics
STAT3	Signal Transducer and Activator of Transcription 3
TAMs	Tumor-Associated Macrophages
TCF7+	Transcription Factor 7 Positive
TGF-β	Transforming Growth Factor-Beta
TIME	Tumor Immune Microenvironment
TME	Tumor Microenvironment
TP53	Tumor Protein 53
Tregs	Regulatory T Cells
UMI	Unique Molecular Identifier

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Conflicts of Interest

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